Amendments to Specification

Please replace the paragraph beginning at page 3, line 16 (spanning pages 3-4), with the following paragraph:

Figure 1A-D depicts a dual fluorescence reporter assay system for RNAi analysis in HeLa cells. (A) Graphical representation of dsRNAs used for targeting GFP mRNA and RFP mRNA. GFP and RFP were encoded by the pEGFP-C1 and pDsRed1-N1 reporter plasmid, respectively. siRNAs were synthesized with 2-nt deoxythymidine overhangs at the 3' end. The position of the first nucleotide of the mRNA target site is indicated relative to the start codon of GFP mRNA or RFP mRNA. The sequence of the antisense strand of siRNA (GFP, SEQ ID NO:2; RFP, SEQ ID NO:6) is exactly complementary to the mRNA target site (GFP, SEQ ID NO:3; RFP, SEQ ID NO:4). (B) Fluorescence images showing specific RNA interference effects in living HeLa cells. Fluorescence in living cells was visualized by fluorescence microscopy at 48 hours post transfection. ~~Panels a and b~~ Left panels, images of mock-treated cells (no siRNA added); middle panels ~~c and d~~, images of GFP siRNA-treated cells; right panels ~~e and f~~, images of RFP siRNA-treated cells. (C) Quantitative analysis of RNAi effects in HeLa cells. Fluorescence emission spectra of GFP and RFP in total cell lysates were detected by exciting at 488 nm and 568 nm, respectively. (D) Kinetics of RNAi effects in HeLa cells. Ratios of normalized GFP to RFP fluorescence intensity over a 66-hour time course. The fluorescence intensity ratio of target (GFP) to control (RFP) protein was determined in the presence of double strand (ds) RNA (~~green~~ black bars) and normalized to the ratio observed in the presence of antisense strand (as) RNA (~~blue~~ white bars). Normalized ratios less than 1.0 indicate specific RNA interference. Maximal RNAi effect occurred at 42 hours post transfection.

Please replace the paragraph beginning at page 4, line 16, with the following paragraph:

Figure 2 depicts the modification of GFP siRNA duplexes. (A) Structure of 5'-N3 (amino group with 3-carbon linker~~, red~~) and 3'-Pmn (puromycin~~, blue~~) modifications. (B) Classification and nomenclature of the modified siRNAs. Sense (top row~~, purple~~) and antisense (botfom row~~,~~

~~black~~) strands of siRNA species are shown with their 5'-N3 (~~red~~ bold) and 3'-Pmn or biotin (~~blue~~ bold) modifications (S-5'-N3, SEQ ID NO:7; AS-5'-N3, SEQ ID NO:8; S-3'-Pmn, SEQ ID NO:9; AS-3'-Pmn, SEQ ID NO:10). A dinucleotide internal bulge structure (~~green~~ bold) was introduced in sense, antisense, or duplex RNAs (AS-3'-biotin, SEQ ID NO:11; S-bulge, SEQ ID NO:12; AS-bulge, SEQ ID NO:13).

Please replace the paragraph beginning at page 4, line 29 (spanning pages 4-5), with the following paragraph:

Figure 4A-B depicts quantitative analysis of RNAi effects in HeLa cells transfected with modified siRNAs. pEGFP-C1 (as reporter), pDsRed1-N1 (as control) plasmids and 50 nM siRNA were cotransfected into HeLa cells by lipofectamine. Cells were harvested at various times after transfection. Fluorescence emission spectra of GFP and RFP in total cell lysates were detected by exciting at 488 nm and 568 nm, respectively. (A) GFP emission spectra of modified siRNAi-treated cells. Emission spectra of GFP in lysates from cells transfected with 5'-modified GFP siRNAs (upper panel), 3'-modified GFP siRNAs (middle panel) and bulge-containing GFP siRNAs (lower panel). For comparison, results from antisense- (as, ~~red~~ dotted line) and unmodified duplex siRNA (ds, ~~black~~ thick line)-treated cells are included in each panel. (B) Ratios of normalized GFP to RFP fluorescence intensity in lysates from modified siRNA-treated HeLa cells over 66 hours. The fluorescence intensity ratio of target (GFP) to control (RFP) fluorophore was determined in the presence of 5'-modified GFP siRNAs (upper panel), 3'-modified GFP siRNAs (middle panel), and bulge-containing GFP siRNAs (lower panel) and normalized to the ratio observed in the presence of antisense strand siRNA. Normalized ratios less than 1.0 indicate specific RNA interference effects. For comparison, results from antisense RNA (as) and duplex (ds) siRNA-treated cells are included in each panel ~~(as, orange bars; ds, yellow bars)~~.

Please replace the paragraph beginning at page 5, line 29 (spanning pages 5-6), with the following paragraph:

Figure 6A-E depicts RNA interference activities of covalently photocross-linked duplex RNA in HeLa cells. (A) Structure of a psoralen derivative, 4'-hydroxymethyl-4,5',8-

trimethylpsoralen (HMT), used to cross-link the duplex RNA. (B) Photocross-linking sites in GFP siRNA. Three preferred sites for psoralen addition to a duplex RNA are shown by ~~cyan~~ bold letters with ~~red~~ bars indicating the C-U cross-links formed by UV irradiation in the presence of HMT. (C) Psoralen photocross-linking of siRNA duplexes. Mixtures of siRNA duplex and psoralen were exposed to UV 360 nm and denatured. Cross-linked and noncross-linked siRNAs were resolved on 20% PAGE containing 7 M urea (lanes 2 and 3). UV-irradiated RNA bands were excised from the gel and purified. Purified cross-linked dsRNA (ds-XL) and noncross-linked dsRNA (ds*) are shown in lanes 6 and 5, respectively. To confirm the nature and purity of the cross-link, a portion of the 360 nm UV-irradiated sample (lane 3) was UV-irradiated at 254 nm. Photoreversal of psoralen cross-linked siRNA resulted in products with similar electrophoretic mobility to the siRNA duplex without HMT treatment (lane 4). (D) Fluorescence images showing RNA interference effects of psoralen photocross-linked siRNAs in living HeLa cells. Purified cross-linked ds siRNA (ds-XL, bottom panels) was cotransfected with reporter pEGFP-C1 and control pDsRed1-N1 plasmids into HeLa cells for dual fluorescence reporter assays. Fluorescence (left panels) and phase contrast (right panels) images of living cells were taken 48 hours post transfection. For comparison, images from noncross-linked ds siRNA (ds*, middle panels) and antisense siRNA (as, top panels) are also shown. (E) GFP emission spectra of psoralen photocross-linked siRNA duplex-treated cells. Cell lysates were prepared from HeLa cells treated with antisense siRNA (as), unmodified UV-irradiated duplex siRNA (ds*) and cross-linked ds siRNA (ds-XL) and analyzed by fluorescence spectroscopy. Fluorescence emission spectra of GFP and RFP were detected by exciting at 488 nm and 568 nm, respectively. GFP emission spectra are shown normalized to RFP expression.

Please replace the paragraph beginning at page 7, line 4, with the following paragraph:

Figure 8A-B depicts fluorescence intensity spectra for extracts of cells transfected with various GFP- and/or RFP-encoding plasmids and, optionally, treated with siRNAs targeting GFP and/or RFP mRNAs. (A) depicts the fluorescence intensity spectra for extracts from cells transfected with dsRed1-N1 versus dsRed2-N1. (B) depicts RNAi of GFP or RFP, left and right panels, respectively.

Please replace the paragraph beginning at page 7, line 9, with the following paragraph:

Figure 9A-B depicts a quantitative analysis of RNAi effects in HeLa cells transfected with modified single-stranded (antisense strand) siRNAs.

Please replace the paragraph beginning at page 7, line 11, with the following paragraph:

Figure 10A-E depicts a quantitative analysis of RNAi effects in HeLa cells transfected with modified duplex siRNAs.

Please replace the paragraph beginning at page 7, line 15, with the following paragraph:

Figure 12A-E depicts the stability of duplex siRNA with 2'-Fluoro uridine and cytidine modification in HeLa cell lysates.

Please replace the paragraph beginning at page 7, line 17, with the following paragraph:

Figure 13A-D depicts a quantitative analysis of RNAi effects of duplex siRNAs with 2'-Fluoro uridine and cytidine modifications, and 2'-Fluoro uridine and cytidine modifications in combination with 2'-deoxy modifications, in HeLa cells (AS-deoxy, SEQ ID NO:14; AS-2'FU,FC, SEQ ID NO:15; AS-2'FU,FC+(9,10,13)dA,dG, SEQ ID NO:16; AS-2'FU,FC+(9-19)dA,dG, SEQ ID NO:17; AS-2'FU,FC+(1-13)dA,dG, SEQ ID NO:18; AS-2'FU,FC+dA,dG, SEQ ID NO:19; S-2'FU,FC, SEQ ID NO:20).

Please replace the paragraph beginning at page 7, line 20, with the following paragraph:

Figure 14A-C depicts a quantitative analysis of RNAi effects of duplex siRNAs with N3-Methyl uridine modifications in HeLa cells (AS-(11)3mU, SEQ ID NO:21; AS-3mU, SEQ ID NO:22).

Please replace the paragraph beginning at page 7, line 22, with the following paragraph:

Figure 15A-C depicts a quantitative analysis of RNAi effects of duplex siRNAs with 2-nucleotide mismatches in the antisense strand in HeLa cells (AS-(18,19) mm, SEQ ID NO:23; AS-(1,2) mm, SEQ ID NO:24).

Please replace the paragraph beginning at page 7, line 32, with the following paragraph:

Figure 19A-B depicts the structures of EGFP siRNA (S, SEQ ID NO:1; AS, SEQ ID NO:2) and the structure and nomenclature of preferred chemical modifications.

Please replace the paragraph beginning at page 39, line 30, with the following paragraph:

The mRNA sequence of CDK9 can be any ortholog of CDK9, such as sequences substantially identical to the *S. cerevisiae*, human, *C. elegans, D. melanogaster,* or mouse CDK9, including but not limited to GenBank Accession Nos. NM_001261 (GI:17017983) ~~(SEQ ID NO:2)~~ (corresponding protein sequence: NP_001252) (human); P50750 (human); NP_570930 (mouse); BA C40824 (mouse); NP_477226 (fruit fly); NP_492906 (*C. elegans*); or NP_492907 (*C. elegans*). The mRNA sequence of CycT1 can be any ortholog of CycT1, such as sequences substantially identical to the *S. cerevisiae*, human, or mouse CycT1, including but not limited to GenBank Accession Nos. AF048730 (GI:2981195) (corresponding protein sequence: AAC39664) (human); NM_001240 (GI:17978465) (corresponding protein sequence: NP_001231) (human); AAN73282 (chimpanzee); NP_033963 (mouse); AAD17205 (mouse); QDQWV9 (mouse); AAM74155 (goat); or AAM74156 (goat).

Please replace the table beginning after page 43, line 4, with the following table:

hCycT1 ds	5'-UCCCUUCCUGAUACUAGAAdTdT-3' SEQ ID NO:25
HcycT1 mm (neg. ctrl)	5'-UCCCUUCCGUAUACUAGAAdTdT-3' SEQ ID NO:26
CDK9 ds	5'-CCAAAGCUUCCCCCUAUAAdTdT-3' SEQ ID NO:27
CDK9 mm (neg. ctrl)	5'-CCAAAGCUCUCCCCUAUAAdTdT-3' SEQ ID NO:28
Spt5 ds	5'-AACTGGGCGAGTATTACATGAdTdT-3' SEQ ID NO:29
Spt5 mm (neg. ctrl)	5'-AACTGGGCGGATATTACATGAdTdT-3' SEQ ID NO:30

Please replace the paragraph beginning at page 68, line 23, with the following paragraph:

To explore the functional anatomy of siRNA in mammalian cells, a dual fluorescence reporter system was established using HeLa cells as a model system. Two reporter plasmids were used: pEGFP-C1 and pDsRed1-N1, harboring enhanced green fluorescent protein (GFP) or coral (*Discosoma spp.*)-derived red fluorescent protein (RFP), respectively. The expression of these reporter genes was under cytomegalovirus promoter control and could be easily visualized by fluorescence microscopy in living cells. The siRNA sequence targeting GFP was from position 238-258 (SEQ ID NO:3) relative to the start codon (AS, SEQ ID NO:2; S, SEQ ID NO:1), and the RFP siRNA sequence was from position 277-297 (SEQ ID NO:4) relative to the start codon (AS, SEQ ID NO:6; S, SEQ ID NO:5) (Figure 1A). Using lipofectamine, HeLa cells were cotransfected with pEGFP-C1 and pDsRed1-N1 expression plasmids and siRNA duplex, targeting either GFP or RFP. Fluorescence imaging was used to monitor GFP and RFP expression levels. As shown in Figure 1B (left panels ~~a and b~~), mock treatment (without siRNA) allowed efficient expression of both GFP and RFP in living cells. Transfection of cells with siRNA duplex targeting GFP (GFP ds) significantly reduced GFP expression (Figure 1B, middle upper panel [c]), but had no effect on RFP expression (Figure 1B, middle lower panel [d]) compared with mock-treated cells ~~(Figure 1B, panels a and b)~~. By contrast, transfection of cells

with siRNA duplex targeted to RFP (RFP ds) (S, SEQ ID NO:5; AS, SEQ ID NO:6) significantly interfered with the expression of RFP, but not GFP (Figure 1B, right upper and lower panels ~~e and f~~).

Please replace the paragraph beginning at page 69, line 8, with the following paragraph:

To quantify RNAi effects, lysates were prepared from siRNA duplex-treated cells at 42 hours post transfection. GFP and RFP fluorescence in clear lysates was measured on a fluorescence spectrophotometer. The peak at 507 nm (Figure 1C, left panel) represents the fluorescence intensity of GFP, and the peak at 583 nm (Figure 1C, right panel) represents the fluorescence intensity of RFP. GFP fluorescence intensity of GFP ds-treated cells (Figure 1C, left panel~~, green line~~) was only 5% of mock-treated ~~(black line)~~ or RFP ds-treated cells ~~(cyan line)~~. In contrast to GFP fluorescence, RFP fluorescence intensity (Figure 1C, right panel) significantly decreased only in cells treated with RFP ds ~~(red line)~~, indicating the specificity of the RNAi effect.

Please replace the paragraph beginning at page 70, line 5, with the following paragraph:

To understand the kinetics of gene suppression and persistence of RNA interference in HeLa cells, lysates were prepared from cells cotransfected with GFP siRNA and dual fluorescence reporter plasmids, pEGFP-C1 and pDsRed1-N1. In this experiment, GFP was the target of the duplex siRNA, while RFP was used as a control for transfection efficiency and specificity of RNA interference. Emission spectra of GFP in cell lysates at various times after transfection (Figure 1G, Supplementary Material) show that siRNA duplex caused an RNA interference effect as early as 6 hours post transfection. This effect gradually increased with time, peaking at 42 hours, then started to decrease at 66 hours (Figure 1G, ~~green~~ thick lines). As a control experiment, GFP expression in the presence of antisense strand was also monitored and showed no RNAi effects (Figure 1G, ~~blue~~ thin lines). Thus, RNA interference can last for at least 66 hours in HeLa cells (Figure 1G, ~~green~~ thick lines).

Please replace the paragraph beginning at page 70, line 17, with the following paragraph:

To quantify the kinetics of RNA interference, the fluorescence intensity ratio of target (GFP) to control (RFP) fluorophore in the presence of siRNA duplex (ds) was measured and normalized it to the ratio observed in the presence of antisense strand siRNA (as). Normalized ratios less than 1.0 indicate specific interference. As shown in Figure 1D, at 6 hours post transfection GFP duplex siRNA (~~green~~ black bars) inhibits 60% of GFP expression compared to antisense strand siRNA (~~blue~~ white bars). RNA interference reached its maximum (92% inhibition) at 42 hours post transfection; only 8% of normal GFP expression was observed in duplex siRNA-treated cells. These results show that RNA interference can suppress target protein expression up to 66 h, although maximum activities were observed at 42-54 h post transfection.

Please replace the paragraph beginning at page 71, line 6, with the following paragraph:

To examine the importance of 5' termini of siRNA in RNA interference in human cells, synthetic siRNAs targeting GFP were modified by using an amino group with a 3-carbon linker (5' N3, Figure 2A) to block their 5' termini. Synthetic siRNAs with this modification lacked a hydroxyl group to be phosphorylated by kinases *in vivo*. This modification could also block access to siRNA by cellular factors that might require recognizing the 5' OH termini. Unmodified siRNA strands were annealed with 5'-modified strands, producing siRNA duplexes with 5' modification at only the sense strand (5'-N3ss/as), at only the antisense strand (ss/5'-N3as) or at both strands (5'-N3ss/5'-N3as) (Figure 2B). RNAi effects of these siRNA duplexes were analyzed in the dual fluorescence reporter system as described in Figure 1. 5' modification of the sense strand had no effect on RNAi activity (Figure 3, compare B and C ~~panels b and~~ c), whereas 5' modification of the antisense strand completely abolished the RNAi effect (Figure 3, D and E ~~panels d and e~~; Figures 4A and 4B, upper panels). HeLa cells transfected with antisense strand (as) siRNA as control showed no RNAi activity (Figure 3A ~~, panel a~~). These results demonstrate that the 5' OH in the antisense strand of the siRNA duplex is an important determinant of RNAi activity in human cells.

Please replace the paragraph beginning at page 71, line 25 (spanning pages 71-72), with the following paragraph:

To determine the effect of 3' OH groups on RNAi activity, siRNA duplexes were synthesized containing a 3' end blocked with 3' puromycin (3'-Pmn, Figure 2A) or biotin instead of 3' OH groups on the overhang deoxythymidine (Figure 2B). These 3' end modifications would block any processing of the siRNA duplex that required a free 3' hydroxyl group. Three combinations of siRNA duplexes were prepared containing 3' puromycin: 3' blocked at only the sense strand (ss3'-Pmn/as), at only the antisense strand (ss/as3'-Pmn), or at both strands (ss3'-Pmn/as3'-Pmn) (Figure 2B). A siRNA duplex containing biotin at the 3'-end of antisense strand (ss/as3'-Biotin) was also prepared. The RNAi activities of these siRNA duplexes were analyzed in our dual fluorescence reporter system. Results of these experiments indicate that a 3' block at either the sense or antisense strand of siRNA duplex had little effect on its RNA interference activity (Figure 3[,]F and I ~~panels f-i~~; Figures 4A and 4B, middle panels). Furthermore, biotin pull out experiments showed that the 3' end biotin groups on the antisense strand were not efficiently removed during RNAi activities in HeLa cells (Figure 5, see below). Modifications could be introduced in the 3' overhangs without affecting siRNA efficacy, suggesting that RNA interference in mammalian cells does not occur through the recently reported RdRP-dependent degradative PCR mechanism (Lipardi et al., 2001; Sijen et al., 2001), which requires a free 3' hydroxyl group.

Please replace the paragraph beginning at page 72, line 25 (spanning pages 72-73), with the following paragraph:

These observations raise two fundamental questions regarding RNAi effects *in vivo*: (1) Is an A-form RNA helix required in the siRNA structure? (2) Is an A-form helix recognized by proteins after the antisense strand of siRNA duplex is hybridized with the target mRNA? To address these questions, three siRNA duplexes were designed containing internal bulge structures in the RNA helices (Figure 2B). The A-form RNA helix has a deep, narrow major groove and a shallow, wide minor groove. More than one nucleotide bulge has been shown to distort RNA helical structures, widening the major groove and enhancing accessibility to its functional groups (Neenhold and Rana, 1995; Weeks and Crothers, 1991; Weeks and Crothers, 1993). 2-nt bulges

were chosen to generate distorted A-form helices in siRNAs. Mutant siRNA were synthesized by introducing two extra nucleotides into the sense (SEQ ID NO:12) or antisense strand (SEQ ID NO:13) of siRNA duplexes. Combining these mutant siRNA strands with original siRNA sequences produced three siRNA duplexes with an internal bulge at only the sense strand (ss-bulge/as), at only the antisense strand (ss/as-bulge), or at both strands (ss-bulge/as-bulge) (Figure 2B). This design of bulge-containing siRNAs could dissect the requirement for the A-form helix at two different steps of RNA interference: 1) siRNA recognition by RISC, and 2) RISC targeting of mRNA via the guiding siRNA. siRNA duplexes with an internal bulge at only the sense strand (ss-bulge/as) caused a structural change in the siRNA duplex (an imperfect A-form) without affecting the complementarity between target mRNA and the antisense strand, which acts as the guiding strand in the RNA interference pathway. RNA interference by these siRNA duplexes was analyzed and quantified in the dual fluorescence reporter system as described above.

Please replace the paragraph beginning at page 73, line 15 (spanning pages 73-74), with the following paragraph:

Surprisingly, the siRNA duplex containing a bulge in its sense strand retained most of its RNA interference activity (Figure 3, compare B and J ~~panels b and j~~; Figures 4A and 4B, lower panels~~, green line and bars~~), indicating that an A-form siRNA helix is not essential for effective RNA interference *in vivo*. However, bulges in the antisense strand or both strands of duplex siRNA completely abolished RNA interference ability (Figure 3K-L~~, panels k and l~~; Figures 4A and 4B, lower panels~~, dark and light blue line and bars~~), indicating that effective RNA interference *in vivo* absolutely requires A-form helix formation between target mRNA and its guiding antisense strand.

Please replace the paragraph beginning at page 74, line 12, with the following paragraph:

These experiments have three key findings. First, biotinylated-siRNA can be isolated from HeLa cells at 6 to 54 hours post transfection (Figure 5, lanes 5-9). The amount of isolated siRNA decreased in a time-dependent manner, indicating the degradation of siRNA *in vivo*. The

dual fluorescence assays showed that RNA interference mediated by 3' end biotinylated siRNA was as effective as unmodified siRNA (Figure 3F and 3B~~, panels f and b~~; Figures 4A and 4B, middle panel). RNA interference is seen as early as 6 hours post siRNA transfection and can be maintained for 42 hours post transfection. The ability to isolate biotin-RNA from cells after RNA interference had been initiated indicates that biotin was not removed from the RNA and rules out the possibility of siRNA 3' OH termini involvement in the RNA interference pathway in human cells.

Please replace the paragraph beginning at page 76, line 5, with the following paragraph:

Unlike the noncross-linked ds siRNA, the two strands of the cross-linked siRNA duplex couldn't separate from each other under denaturing conditions so that the cross-linked siRNA duplex showed characteristically retarded mobility in polyacryalmide gel electrophoresis (PAGE) containing 7M urea (Figure 6C). Cross-linking efficiency depended on the psoralen concentration (Figure 6C, lanes 2 and 3). To further verify the presence of cross-links in the RNA helix and rule out the possibility of only monoadduct formation, the psoralen cross-links were irradiated with short wave UV (254 nm), which showed photoreversal of the cross-linked bonds (Figure 6C, lane 4). The cross-linked siRNA duplex (Figure 6C, lane 3, upper band) was excised from the gel and purified. As control, the noncross-linked siRNA that was irradiated with long wave UV (360 nm) (Figure 6C, lane 3, lower band) was also purified by the same method. The structures of the purified noncross-linked and psoralen cross-linked siRNA duplexes were confirmed by PAGE containing 7M urea (Figure 6C, lanes 5 and 6). Fluorescence imaging of living cells treated with cross-linked siRNA duplex showed that the siRNA duplex's inability to separate on PAGE did not completely abolish its RNA interference activity (Figure 6D, ds-XL). Quantitative analysis of GFP fluorescence intensity indicated that cross-linked siRNA retained 30% of its RNAi activity (Figure 6E, ds-XL ~~blue line~~). These results demonstrate that a complete unwinding of the siRNA duplex is not required for gene silencing *in vivo* (see Discussion).

Please replace the paragraph beginning at page 80, line 24 (spanning pages 80-81), with the following paragraph:

In an improved dual fluorescence reporter assay, EGFP-C1 encoded enhanced green fluorescence protein (GFP), while DsRed2-N1 encoded red fluorescence protein (RFP2) as described above. Using lipofectamine, HeLa cells were cotransfected with pEGFP-C1 and pDsRed2-N1 expression plasmids and siRNA duplex, targeting either GFP or RFP. To quantify RNAi effects, lysates were prepared from siRNA duplex-treated cells at 42 hr posttransfection. GFP and RFP fluorescence in clear lysates was measured on a fluorescence spectrophotometer. The peak at 507 nm (Figure 8B, left panel) represents the fluorescence intensity of GFP, and the peak at 583 nm (Figure 8B, right panel) represents the fluorescence intensity of RFP. GFP fluorescence intensity of GFP ds-treated cells (Figure 8B, left panel, ~~green~~ medium line) was only 5% of mock-treated (~~black~~ thin line) or RFP ds-treated cells (~~blue~~ thick line). In contrast to GFP fluorescence, RFP fluorescence intensity (Figure 8B, right panel) significantly decreased only in cells treated with RFP ds (~~red~~ thick line), indicating the specificity of the RNAi effect.

Please replace the paragraph beginning at page 85, line 5, with the following paragraph:

To address whether increased stability seen with modified siRNAs prolonged the duration of RNAi *in vivo*, RNAi, induced by unmodified and 2'FU, FC modified double-stranded EGFP siRNAs, was assayed in the dual fluorescence reporter assay over a period of 120 h (Figure 11). The fluorescence intensity ratio of target (GFP) to control (RFP) protein was determined in the presence of unmodified double-strand (ds) RNA (~~blue~~ hatched bars) and duplex siRNA with 2'-Fluoro uridine and cytidine modification (ds-2'FU, 2'FC, ~~cyan~~ black bar) and normalized to the ratio observed in the presence of Mock treated cells (~~red~~ white bars). A normalized ratio of less than 1.0 indicates specific RNA interference.

Please replace the paragraph beginning at page 86, line 23 (spaning pages 86-87), with the following paragraph:

Results from experiments demonstrating similar results are depicted in Figure 12D and 12E. Figure 12D shows the stability of the various 2'FU, FC modified siRNAs as compared to wild type siRNAs over time. Wild type double-stranded siRNAs showed a steady loss of intact siRNAs over the course of the experiment, with only ~7% of the original concentration of intact siRNAs remaining after 1 h in extract (Figure 12D; DS, white triangles ~~dark blue line~~). Intact modified or unmodified single stranded antisense siRNAs were quickly lost over the time course and were virtually undetectable by 30 min in extract (Figure 12D; white squares and white circles with dashed line ~~black and red lines~~). In contrast, double-stranded siRNAs with 2'FU, FC modifications in either the antisense strand or both strands remained predominantly intact over the course of the experiment with ~68 or ~81%, respectively, of the original siRNA population remaining intact throughout the duration of the experiment (Figure 12D; white circles and black circles with solid lines ~~green and light blue lines~~). These results indicated that the 2'FU, FC modifications did indeed increase the stability of the siRNAs upon exposure to extract and that having these modifications in both strands provided the siRNAs with the most stability.

Please replace the paragraph beginning at page 87, line 5, with the following paragraph:

In a similar experiment, the stability of P-S modified EGFP siRNAs was evaluated. Unmodified, doubled-stranded antisense siRNAs showed about the same rate of siRNA loss as described in the above experiment (Figure 12E; white squares ~~dark blue lines~~). However, P-S modified single-stranded antisense siRNAs showed a markedly increased rate of stability over the course of the experiment, showing ~63% of the original siRNAs remaining intact after 1 h in extract as compared to 0% intact for single-stranded unmodified antisense siRNAs (Figure 12E; short- and long-dashed lines ~~black and red lines~~). Stability of double-stranded siRNAs with P-S modifications in both strands was comparable to the stability seen with the modified single-stranded antisense strand with ~63% of the originally siRNA population remaining intact after 1 h (Figure 12E; black circles ~~light blue lines~~). Double-stranded siRNAs with P-S modifications in only the antisense strand showed weaker but still significant stability with ~42% of the original

siRNA population remaining intact through to 1 h in extract (Figure 12E; white circles ~~green lines~~). These results showed that the P-S modifications increased the stability of the siRNAs and most notably, increased the stability of both single and double stranded siRNAs.

Please replace the paragraph beginning at page 88, line 8, with the following paragraph:

Modified siRNA duplexes with modifications in the antisense strand at the 2' position of the sugar unit are set forth in Figure 13A and consisted of the following: 2'-hydroxyl wild type (DS) (S, SEQ ID NO:1; AS, SEQ ID NO:2), 2'-deoxy modified as siRNAs (SS/AS-Deoxy) (AS-deoxy, SEQ NO:14), 2'-Fluoro U and C modified as siRNAs (SS/AS-2'FU,FC) (AS-2'FU,FC, SEQ ID NO:15), 2'-Fluoro U and C and 2'-deoxy A and G at positions 9, 10, and 13 modified as siRNAs (SS/AS-2'FU,FC + (9,10,13) dA, dG) (AS-2'FU,FC+(9,10,13)dA,dG, SEQ ID NO:16), 2'-Fluoro U and C and 2'-deoxy A and G at positions 9-19 modified as siRNAs (SS/AS-2'FU,FC + (9-19) dA, dG) (AS-2'FU,FC+(9-19)dA,dG, SEQ ID NO:17), 2'-Fluoro U and C and 2'-deoxy A and G at positions 1-13 modified as siRNAs (SS/AS-2'FU,FC + (1-13) dA, dG) (AS-2'FU,FC+(1-13)dA,dG, SEQ ID NO:18), and 2'-Fluoro U and C and 2'-deoxy A and G modified as siRNAs (SS/AS-2'FU,FC, dA,dG) (AS-2FU,FC,dA,dG, SEQ ID NO:19). The hypothetical cleavage site on the target mRNA is also depicted. The data from cell treated with duplex siRNA with modified antisense strands are set forth in Figure 13B. A normalized ratio of less than 1.0 indicates a specific RNA interference effect. For comparison, results from unmodified duplex siRNA (ds, lanes 2-6)-treated cells are included.

Please replace the paragraph beginning at page 89, line 3, with the following paragraph:

Figure 13C depicts siRNA duplexes with modifications in both strands at the 2' position of the sugar unit, and consisted of the following: 2'-hydroxyl wild type (DS, lanes 2-6) (S, SEQ ID NO:1; AS, SEQ ID NO:2), 2'-deoxy modified as siRNAs (SS/AS-Deoxy, lanes 7-15) (AS-deoxy, SEQ ID NO:14), 2'-Fluoro U and C modified in both strands (SS-2'FU,FC /AS-2'FU,FC, lanes 16-24) (S-2'FU,FC, SEQ ID NO:20; AS-2'FU,FC, SEQ ID NO:15), 2'-Fluoro U and C modified in both strands and 2'-deoxy A and G at positions 9, 10, and 13 within the antisense

strand (SS-2'FU,FC /AS-2'FU,FC + (9,10,13) dA, dG, lanes 25-33) (S-2'FU,FC, SEQ ID NO:20; AS-2'FU,FC+(9,10,13)dA,dG, SEQ ID NO:16), 2'-Fluoro U and C modified in both strands and 2'-deoxy A and G at positions 9-19 within the antisense strand (SS-2'FU,FC /AS-2'FU,FC + (9-19) dA, dG, lanes 34-42) (S-2'FU,FC, SEQ ID NO:20; AS-2'FU,FC +(9-19)dA,dG, SEQ ID NO:17), 2'-Fluoro U and C modified in both strands and 2'-deoxy A and G at positions 1-13 within the antisense strand (SS-2'FU,FC /AS-2'FU,FC + (1-13) dA, dG, lanes 43-51) (S-2'FU,FC, SEQ ID NO:20; AS-2'FU,FC +(1-13)dA,dG, SEQ ID NO:18), and 2'-Fluoro U and C modified in both strands and 2'-deoxy A and G within the antisense strand (SS-2'FU,FC /AS-2'FU,FC, dA, dG, lanes 52-60) (SS-2'FU,FC, SEQ ID NO:20; AS-2'FU,FC,dA,dG, SEQ ID NO:19). Results from cells treated with duplex siRNA with modifications in both strands as set forth in Figure 13C are depicted in Figure 13D and table 1, rows 6, 8, 30, 32.

Please replace the paragraph beginning at page 90, line 15, with the following paragraph:

pEGFP-C1 (as reporter), pDsRed2-N1 (as control) plasmids and various amount of modified siRNA were cotransfected into HeLa cells by lipofectamine. Cells were harvested at 42h after transfection. Fluorescence intensity of GFP and RFP in total cell lysates were detected by exciting at 488 and 568 nm, respectively. The fluorescence intensity ratio of target (GFP) to control (RFP) fluorophore was determined in the presence of modified siRNAs and normalized to the ratio observed in the mock treated cells. Figure 14C depicts the results from cells treated with duplex siRNA having 3mU modifications within the entire antisense strand (SS/AS-3mU, lanes 7-15), 3mU modifications within the entire antisense strand (SS/AS-3mU, lanes 7-15) (S, SEQ ID NO:1; AS-3mU, SEQ ID NO:22), 3mU modifications within the entire antisense strand and 2'-Fluoro modifications at uridine and cytidine bases within the sense strand (SS-2'FU, FC/AS-3mU, lanes 16-24) (S-2'FU, FC, SEQ ID NO:20; AS-3mU, SEQ ID NO:22), and 3mU modification at position 11 within the antisense strand (SS/AS-(11)-3mU, lanes 25-33) (S, SEQ ID NO:1; AS-(11)3mU, SEQ ID NO:21). The modified siRNA duplexes were prepared by annealing modified antisense strand containing single or multiple 3mU modifications with unmodified sense strand (SS/AS-(11)-3mU and SS/AS-3mU) or sense strand having 2'-Fluoro

modifications (SS-2'FU, FC/AS-3mU). For comparison, results from cells treated with unmodified duplex siRNA (ds, lane 2-6) are also shown. 3MU modified EGFP siRNAs introduced into Hela cells completely abolished RNAi (Figure 14C, Table 1, rows 25). RNAi was also abolished if only one 3MU modification was introduced specifically at U11 of the antisense strand, which is one of the nucleotides that base pairs with A248 of the target EGFP mRNA cleavage site (Figure 14B and 14C, Table 1, row 26). These results indicated that disrupting the functional groups of the major groove of the A-form helix formed by the antisense strand and its target mRNA specifically at the cleavage site inhibited RNAi. These data also suggested that the major groove was required for mediating RNAi and for RNA-RISC* interactions that subsequently lead to mRNA cleavage.

Please replace the paragraph beginning at page 91, line 24 (spanning pages 91-92), with the following paragraph:

This Example directly tests the model set forth above and demonstrates an aysmmetric requirement for duplex siRNA structure in RNA interference *in vivo*. To test this hypothesis, EGFP siRNAs with mismatched base pairs at either the 5' (nt 1, 2) (AS-(1,2)mm, SEQ ID NO:24) or 3' (nt 18, 19) (AS-(18,19)mm, SEQ ID NO:23) ends were introduced into the antisense strand (Figure 15B). pEGFP-C1 (as reporter), pDsRed2-N1 (as control) plasmids and various amount of modified siRNA were cotransfected into HeLa cells by lipofectamine. Cells were harvested at 42h after transfection. Fluorescence intensity of GFP and RFP in total cell lysates were detected by exciting at 488 and 568 nm, respectively. The fluorescence intensity ratio of target (GFP) to control (RFP) fluorophore was determined in the presence of modified siRNAs and normalized to the ratio observed in the mock treated cells. Figure 15C depicts results from cells treated with duplex siRNA having mismatches located at the 3' end [SS/AS-(18,19)mm, lanes 7-14] or 5' end [SS/AS-(1,2)mm, lanes 15-22] of the antisense strand. For comparison, results from unmodified duplex siRNA-treated cells are also shown (ds, lane 2-6). siRNAs with mismatches near the 5' end of the antisense strand showed only ~35% inhibition in the dual fluorescence reporter assay whereas mismatches at the 3' end retained a significant level of gene silencing at ~77% (Figure 15C; Table 1, rows 27-28). These results strongly indicated

that the integrity at the 5' end of the antisense strand in the duplex was functionally more important than the 3' end.

Please replace the paragraph beginning at page 106, line 17, with the following paragraph:

For mapping of the target RNA cleavage, a 124 nucleotide transcript was generated corresponding to the EGFP between positions 195 and 297 relative to the start codon followed by the 21 nucleotide complement of the SP6 promoter sequence. The 124 nucleotide transcript was amplified from template EGFP-C1 by PCR using the 5' primer, GCC*T***AATACGACTCACTATAG**GACCTACGGCGTGCAGTGC (T7 promoter in bold ~~underlined~~) (SEQ ID NO:33), and the 3' primer, TTG**ATTTAGGTGACACTATAG**ATGGTGCGCTCCTGGACGT (SP6 promoter in bold ~~underlined~~) (SEQ ID NO:34). The his-tagged mammalian capping enzyme was expressed in *E. coli* from a plasmid generously provided by Dr. Stewart Shuman and was purified to homogeneity. Guanylyl transferase labeling was performed by incubating 1 nmole transcript with 100 pmole his-tagged mammalian capping enzyme in a 100μl capping reaction containing 50mM Tris-HCl (pH 8.0), 5mM DTT, 2.5 mM $MgCl_2$, 1U/l RNasin Rnased inhibitor (promega) and [α-^{32}P]GTP at 37°C for 1hr. The reaction was chased for 30 minutes by supplementing with unlabeled GTP to a concentration of 100μM. Cap-labeled target RNA was resolved on a 10% polyacrylamide-7M urea gel and was purified.

Please replace the paragraph beginning at page 107, line 20 (spanning pages 107-108), with the following paragraph:

RNAi was used to inhibit hCycT1 and CDK9 expression in cultured human (HeLa) cell lines. The short interfering RNA (siRNA) sequence targeting hCycT1 (SEQ ID NO:25) was from position 347 to 367 relative to the start codon, and the CDK9 siRNA sequence (SEQ ID NO:27) was from position 258 to 278 relative to the start codon. Using lipofectamine, HeLa cells were transfected with hCycT1 or CDK9 siRNA duplex, targeting either hCycT1 or CDK9. To analyze RNAi effects, lysates were prepared from siRNA duplex-treated cells at various times after transfection. Western blot experiments were carried out using anti-hCycT1 and anti-CDK9

antibodies. Briefly, HeLa cells were transfected with double-stranded (ds) siRNAs targeting RFP, hCycT1, or CDK9. Cells were also transfected with mutant siRNAs (hCycT1 mismatch (SEQ ID NO:26) or CDK9 mismatch (SEQ ID NO:28)) having 2 nucleotide mismatches between the target mRNA and the antisense strand of siRNA at the hypothetical cleavage site of the mRNA. Cells were harvested at various times post transfection, their protein content resolved on 10% SDS-PAGE, transferred onto PVDF membranes, and immunoblotted with antibodies against hCycT1 and CDK9. Analysis of immunoblotting experiments reveals that the siRNA targeting hCycT1 inhibited hCycT1 protein expression. siRNA targeting CDK9 was similarly specific against CDK9 expression. This RNAi effect depended on the presence of a 21-nt duplex siRNA harboring a sequence complementary to the target mRNA, but not on single stranded antisense strand siRNAs nor on an unrelated control siRNA, which targeted a coral (*Discosoma spp.*)-derived red fluorescent protein (RFP). As a specificity control, cells were also transfected with mutant siRNAs (mismatched siRNA) of hCycT1 or CDK9, which have two nucleotide mismatches between the target mRNA and the antisense strand of siRNA at the putative cleavage site of the mRNA. Mutant siRNAs showed no interference activity, indicating the specificity of the RNAi effect. Thus, the siRNAs of the present invention specifically silence the subunits of P-TEFb in HeLa cells.

Please replace the paragraph beginning at page 115, line 16 (spanning pages 115-116), with the following paragraph:

To inhibit hSpt5 expression in a cultured human cell line using RNAi, siRNA targeting an hSpt5 sequence from position 407 to 427 relative to the start codon was designed (SEQ ID NO:32). Magi cells were then transfected with hSpt5 duplex siRNA using Lipofectamine (Invitrogen). To evaluate the effects of hSpt5 RNAi, total cell lysates were prepared from siRNA-treated cells harvested at various time points after transfection. hSpt5 mRNA or protein levels were then analyzed by RT-PCR or western blot using anti-hSpt5 antibodies, respectively. These experiments showed that cells transfected hSpt5 siRNA had significantly lowered hSpt5 mRNA and protein expression, indicating that RNAi of hSpt5 had occurred successfully. This knockdown effect was dependent on the presence of a 21-nt siRNA duplex harboring a sequence

complementary to the mRNA target. Mock-treated (no siRNA), single-stranded antisense hSpt5 siRNA, mismatched hSpt5 duplex siRNA (SEQ ID NO:33), containing two nucleotide mismatches between the target mRNA and siRNA antisense strand at the putative cleavage site of the target mRNA did not affect hSpt5 mRNA or proteins levels. This suggested that hSpt5 knockdown was specific to duplex siRNA exactly complementary to the hSpt5 mRNA target. In evaluating either mRNA or protein levels, human Cyclin T1 (hCycT1) was used as an internal control, showing that the effects of hSpt5 siRNA were specific to hSpt5 and did not effect hCycT1 mRNA or protein levels. Taken together, these results suggested that hSpt5 knockdown was sequence specific and led to significantly decreased hSpt5 mRNA and proteins levels.

Please replace the paragraph beginning at page 120, line 26 (spanning pages 120-121), with the following paragraph:

The targeted region in the mRNA, and hence the sequence of CycT1 or CDK9-specific siRNA duplexes was designed following the guidelines provided by Dharmacon (Lafayette, CO). Briefly, starting 100 bases downstream of the start codon, the first AA dimer was located and the next 19 nucleotides were then recorded following the AA dimer. Criteria were set such that the guanosine and cytidine content (G/C content) of the AA-N19 21 base-sequence must be less than 70% and greater than 30%. The search continued downstream until the conditions were met. The 21-mer sequence was subjected to a BLAST search against the human genome/NCBI EST library to ensure only the desired gene was targeted. The siRNA sequence targeting hCycT1 was from position 347-367 relative to the start codon. The siRNA sequence targeting CDK9 was from position 258-278 relative to the start codon. siRNA sequences used in our experiments were: hCycT1 ds (5'-UCCCUUCCUGAUACUAGAAdTdT-3') (SEQ ID NO:[3]25); hCycT1 mm (5'-UCCCUUCC**GU**AUACUAGAAdTdT-3') (SEQ ID NO:[4]26); CDK9 ds (5'-CCAAAGCUUCCCCCUAUAAdTdT-3') (SEQ ID NO:[5]27); CDK9 mm (5'-CCAAAGCU**CU**CCCCUAUAAdTdT-3') (SEQ ID NO:[6]28); CDK7 ds (5'-UUGGUCUCCUUGAUGCUUUdTdT-3') (SEQ ID NO:[17]31); Tat ds (5'-GAAACGUAGACAGCGCAGAdTdT-3') (SEQ ID NO:[18]32); GFP ds (5'-GCAGCACGACUUCUUCAAGdTdT-3') (SEQ ID NO:[19]1); and RFP ds (5'-

GUGGGAGCGCGUGAUGAACdTdT-3') (SEQ ID NO:[20]5). Bold ~~Underlined~~ residues represent the mismatched sequence to their targets.

Please replace the paragraph beginning at page 122, line 2, with the following paragraph:

The targeted region in the mRNA, and hence the sequence of Spt5-specific siRNA duplexes, was designed following the guidelines provided by Dharmacon (Lafayette, CO). Briefly, beginning 100 bases downstream of the start codon, the first AA dimer was located and then the next 19 nucleotides following the AA dimer were recorded. Ideally, the guanosine and cytidine content (G/C content) of the AA-N19 21 base-sequence would be less than 70% and greater than 30%. The search was continued downstream until the conditions were met. The 21-mer sequence was subjected to a BLAST search against the human genome/NCBI EST library to ensure only the desired gene was targeted. The siRNA sequence targeting hSpt5 was from position 407-427 relative to the start codon. siRNA sequences used in the experiments described herein were: hSpt5ds (5'- AACTGGGCGAGTATTACATGAdTdT-3') (SEQ ID NO: [8]29); h Spt5 mm (5'- AACTGGGCG**GA**TATTACATGAdTdT-3') (SEQ ID NO: [9]30); Tat ds (5'-GAAACGUAGACAGCGCAGAdTdT-3') (SEQ ID NO: [18]32); GFP ds (5'-GCAGCACGACUUCUUCAAGdTdT-3') (SEQ ID NO: [19]1); and RFP ds (5'-GUGGGAGCGCGUGAUGAACdTdT-3') (SEQ ID NO: [20]5). Bold ~~Underlined~~ residues represent the sequences mismatched to their targets.